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(71) Applicant *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPT. OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, 6011 Executive Blvd., Suite 325, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: RUIZ, Margaret [US/US]; 7843 Nevois Lane, East Carondelet, IL 62240 (US). KINTER, Audrey [US/US]; 6200 Dunrobbin Dr., Bethesda, MD 20816 (US). DYBUL, Mark [US/US]; 1730 New Hampshire Ave., Apt. 4, Washington, DC 20009 (US). CATANZARO, Andrew [US/US]; 1733 Kilbourn, Apt. C, Washington, DC 20010 (US). FAUCI, Andhony, S. [US/US]; 3012 43rd St., Washington, DC 20016 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson and Bear, LLP, 620 Newport Center Drive, 16th Floor, Newport Beach, CA 92660 (US).

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(54) Title: METHOD OF IN VITRO T CELL DIFFERENTIATION OF CD34+ PROGENITOR CELLS

(57) Abstract: The invention relates to methods of generating T cell from primate hematopoietic T cell progenitor cells in the presence of lymph node material and various cytokine cocktails. The mature T cells may be used to treat a variety of immunodeficiency disorders. Methods of generating genetically modified T cells are also discussed. Further, methods of determining effective T cell progenitor cell differentiation-promoting cocktails are discussed.

METHOD OF IN VITRO T CELL DIFFERENTIATION OF CD34+ PROGENITOR CELLS

Background of the Invention

The present invention relates generally to the field of T cell differentiation, and more specifically, to systems useful for inducing the growth and differentiation of T cells.

The hematopoietic stem cell is the progenitor for all of the leukocytic and erythrocytic blood cells, including the lymphocytic and myelomonocytic lineages, as well as other types of cells such as osteoclasts. These cells provide an enormous range of functions and are believed to be the only cells that are self-regenerating and maintain their pluripotent potential during the life of the host. During embryogenesis, hematopoietic stem cells originate in coordinate waves within the extra-embryonic yolk sac and the fetal aortagonadomesonephros (AGM). Later, during definitive hematopoiesis, pluripotent stem cells populate the fetal liver and the bone marrow. Pluripotent hematopoietic stem cells migrate from these regions to the thymus and serve as the precursors for T lymphocytes. Postnatally, pluripotent stem cells account for approximately 1% of the low density nucleated bone marrow cells; these provide a renewable source of stem cells to regenerate the hematopoietic system during adult life. The differentiation of hematopoietic cells is dependent on a variety of factors, including the stromal microenvironment and differentiation factors.

The differentiation of T cells occurs through a series of discrete developmental stages characterized by specific T cell markers. In humans, the pluripotent progenitor cell has been shown to express high levels of the CD34 marker on the cell surface. As the pluripotent cells develop and commit to either the lymphoid, monomyeloid, or erythroid cells, the level of CD34 expression decreases.

The pluripotent hematopoietic $CD34^+$ T cell progenitor cell does not express the T cell markers CD3, CD4, and CD8. Differentiating $CD34^+$ cells progress from Stage 0 (the undifferentiated state), to Stages 1, 2, 3, and 4, as shown in Figure 1. In Stage 0, cells are double negative for CD4 and CD8 and appear in the lower left quadrant. The CD34+ progenitor cells ($CD34^+CD3^-CD4^-CD8^-$) subsequently express lymphocyte specific makers and lose the expression of CD34 as they differentiate ($CD34^-CD3^+CD4^+CD8^+$)(Stages 1, 2, and 3). Developing T cells become double positive for the expression of CD4 and CD8 and express variable levels of CD3 on the surface of the cell. Differentiation continues after the appropriate selection of receptors such that mature T cells express either CD4 ($CD3^+CD4^+CD8^-$) or CD8 ($CD3^+CD4^-CD8^+$) alone with the CD3 receptor. Single positive mature T cells which have completed Stage 4 are shown in the lower right and upper left quadrant respectively.

Studies of human lymphopoiesis have been done largely in the xenogeneic SCID-hu mouse model. Alternately, murine thymic explant studies (Plum, J., *et al.*, *Blood* 84:1587-1593, 1994) and simian thymic monolayer cultures (Rosenzweig, M., *et al.*, *Blood* 87(10): 4040-4048, 1996) have been employed. Human thymic explant studies (U.S. Patent 5,677,139) have also been utilized. All of the techniques require the use of allogeneic thymic stromal cells in order to culture or differentiate adult stem cells. T cells that develop in a xenogeneic environment may not demonstrate appropriate host MHC restriction.

Summary of the Invention

The present invention relates to the generation of a population of T cells derived from a population of hematopoietic T cell progenitor cells. For example, one embodiment of the invention is a method for *in vitro* T cell production, comprising the steps of culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell.

The invention is contemplated as useful for treating individuals with damaged immune systems. For example, one embodiment of the invention is a method of treating a subject having an immune disorder, comprising the steps of culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell to produce a descendent cell, and administering a therapeutically effective amount of the progenitor cell or the descendent of the progenitor cell to the subject. Another therapeutic embodiment of the invention is a method of treating an immune disorder in a subject, comprising administering a therapeutically effective amount of a cytokine to a lymph node in the subject, wherein the cytokine comprises at least IL-2 and IL-6.

Another embodiment of the invention is a method for modifying a hematopoietic stem cell to produce a descendent cell containing a nucleotide sequence of interest. The method comprises the steps of culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell to produce a descendent cell, contacting the primate hematopoietic T cell progenitor cell and the lymph node cells with an expression vector comprising a nucleotide sequence of interest, wherein the contacting is for a period of time sufficient for the virus to enter the hematopoietic T cell progenitor cell.

There are other embodiments contemplated by the invention. For example, one embodiment contemplated by the invention is a method for testing the effect of an agent on a hematopoietic cell, comprising the steps of co-culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell, contacting the hematopoietic T cell progenitor cell with an agent, and comparing the differentiation or growth of the hematopoietic T cell progenitor cell with the differentiation or growth of a control cell not contacted with the agent.

Still another embodiment of the present invention is a kit comprising a container containing a cryopreserved lymph node cell and instructions for culture of the lymph node cell with a hematopoietic T cell progenitor cell.

Brief Description of the Drawings

FIGURE 1 shows a graphic representation of the stages of T cell progenitor cell development as such cells would appear during FACS analysis.

FIGURE 2 shows FACS graphs A through O that depict a number of results concerning various growth conditions used in the methods described herein. T cell surface markers CD3, CD4, and CD8, and other markers are followed in this series of graphs. A number of fluorescent probes are used in these experiments. For example, CY (CY5), FITC, PE, QR, and TCR-ab FITC are all used.

FIGURE 3 is a three dimensional FACS representation of the expression of CD3, CD4 and CD8 in a lymph node cocultured with $CD34^+$ progenitor cells obtained from a patient with end stage HIV utilizing the methods described herein.

FIGURE 4 is a graphic representation of various populations of cells and their response to mitogen stimulation. Mitogens used were (PHA) and the response to mitogen is represented as the Stimulation Index, which is calculated by quantitating the amount of tritiated thymidine incorporated by the assayed cells. The Stimulation Index is the quotient of the total number of counts divided by the number of counts assayed from an unstimulated control. The abbreviation LNMC means lymph node mononuclear cells.

FIGURE 5 is a graphic representation of various populations of cells obtained from an end stage HIV+ patient utilizing the methods described herein and testing responses to allo-antigen stimulation. The lymphocyte response to stimulation by allo-antigen is MHC-restricted but it is not antigen specific at the T cell receptor (TCR).

FIGURE 6 is a graphical representation of various populations of cells and their response to antigen stimulation when the antigens were SE-A and SE-B.

Detailed Description of the Preferred Embodiment

A method is provided for *in vitro* T cell production, wherein a lymph node tissue fragment is cultured *in vitro* with a primate hematopoietic $CD34^+CD3^-$ progenitor cell. "Hematopoietic T cell progenitor cells" are those cells capable of differentiating into mature T cells. T cell progenitor cells include stem cells. A "stem cell" is a pluripotent cell that is capable of self-renewal and differentiation into all myeloid and lymphoid cell lineages, including T cells. A pluripotent stem cell gives rise to progeny of all defined hemato-lymphoid lineages; and limited numbers of these cells are capable of fully reconstituting an immuno-compromised host in all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell by cell renewal.

The T cell progenitor cells which are employed may be fresh, frozen, or have been subject to a treatment prior to culture. They may be fetal, neonate, adult, and can be isolated from sources including fetal liver, bone marrow, umbilical cord blood, or peripheral blood. In addition, T cell progenitor cells include peripheral blood stem cells obtained from the peripheral blood of subjects who have been treated with chemotherapeutic agents and/or cytokines (*e.g.*, G-CSF or GM-CSF) to increase hematopoietic T cell progenitor cells circulating in the peripheral blood. In one embodiment, the hematopoietic T cell progenitor cells are derived from a mammal. In another embodiment, the T cell

progenitor cells are derived from a primate. The primate can be a human or a non-human primate. Non-human primates include, but are not limited to *Aotus triviruius* (owl monkey), *Ateletes geoffroy* (spider monkey), *Cebus Albifroms* (white and brown capuchin monkey), *Cercopitecus aethiops* (African green monkey), *Calitrix jacchus* (common marmoset monkey), *Macaca mulatta* (rhesus monkey), *Macaca fascicularis* (crab eater (cynomolgus) monkey), *Pan traoglodytes* (chimpanzee), *Papio anubis* (baboon), *Saguinus fuscicollis* (tamarin monkey), *Salmiri sciurus* (squirrel monkey).

The T cell progenitor cells can be isolated from a normal subject or a subject with a disorder of the immune system. Immune disorders include "immunodeficiency disorders," wherein a general deficiency of T cells exists or a deficiency of specific populations of T cells exists. An example of an acquired immunodeficiency disorder is AIDS, an example of a congenital T cell immunodeficiency is DiGeorge's Syndrome (Complete Athymia), or an acquired immunodeficiency secondary to chemotherapy. Immune disorders include "immunoproliferative" disorders, wherein an increase in the overall numbers of lymphocytes or a specific population of T cells exists. An example of an immunoproliferative disorder is a lymphoma. In this regard, a subject's own cells can be removed, expanded and/or differentiated, and optionally genetically altered.

In one embodiment, the subject is infected with an immunodeficiency lentivirus. The term "lentivirus" is used in its conventional sense to describe a genus of viruses containing reverse transcriptase. The lentiviruses include the "immunodeficiency viruses" which include human immunodeficiency virus (HIV) type 1 and type 2 (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV). In the absence of effective therapy, most individuals infected with a human immunodeficiency virus develop acquired immune deficiency syndrome (AIDS) and succumb to either opportunistic infections and malignancies resulting from the deterioration of the immune system and the direct effects of the virus. $CD34^+$ cells isolated from patients infected with HIV have been shown to be able to reconstitute an allogeneic thymus, without evidence of latent HIV. (U.S. Patent 5,677,139 and *J Immunol* 161(8):4169-76 (1998)).

Hematopoietic T cell progenitor cells at various stages of differentiation may be used, although it is preferable to use progenitor cells in very early stages of differentiation. For example, $CD34^+$ progenitor cells can be used. Hematopoietic stem cells are cells that are both self-renewing and pluripotent. These cells constitute approximately 0.01% of low density nucleated bone marrow cells. Pluripotent hematopoietic stem cells express a high level of CD34 antigen. These cells eventually develop and commit to the erythroid, monomyeloid, or lymphoid cells, including T cells. $CD34^+$ T cell progenitor cells do not express the T cell markers CD3, CD4, and CD8 on the cell surface. During differentiation into mature T cells, the cells pass through an intermediate state where the cells express variable levels of CD3, CD4 and CD8 (termed $CD3^+CD4^+CD8^+$, or double positive cells for $CD4^+CD8^+$). These $CD3^+CD4^+CD8^+$ double positive hematopoietic T cell progenitor cells are immature T cells at an intermediate stage of differentiation which have lost expression of CD34. These cells subsequently mature into cells that are $CD34^-CD3^+$ (bright), and further express only one of the CD4 or CD8 cell surface markers. Thus, mature T cells are either positive for CD4 or CD8 which is expressed with the CD3 receptor as $CD3^+CD4^+$ or $CD3^+CD8^+$.

The manner in which the T cell progenitor cells are separated from other cells of the hematopoietic or other lineage is not critical to this invention. In one nonlimiting example, the stem cells may be separated as described in U.S. Patent No. 5,061,620 or U.S. Patent 5,799,944. A "substantially enriched" hematopoietic T cell progenitor cell population refers to a substantially homogeneous population of hematopoietic T cell progenitor cells that are substantially free from other cells with which they are naturally associated. As described in U.S. Patent Nos. 5,061,620, and 5,766,944, a substantially enriched population of stem cells may be obtained by selective isolation of cells free of markers associated with differentiated cells, while displaying epitopic characteristics associated with the stem cells. Usually the population of hematopoietic T cell progenitor cells will comprise less than about 10% lineage committed cells, more usually less than about 5% lineage committed cells. The hematopoietic T cell progenitor cells are characterized by both the presence of markers associated with specific epitopes identified by antibodies and the absence of certain markers as identified by the lack of binding of specific antibodies. At such time as a specific marker is identified for hematopoietic T cell progenitor cells, binding of an antibody to such a marker may provide the desired composition.

A large proportion of the differentiated or lineage committed cells may be removed initially by using a relatively crude separation technique, where the major cell populations of the hematopoietic system, such as the lymphocytic and myelomonocytic lineages, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed. If desired, a prior separation may be employed to remove erythrocytes, by employing ficoll-hypaque (Sigma, St. Louis, MO) separation. The gross separation may be achieved using magnetic beads, cytotoxic agents, affinity chromatography, panning, or the like. Antibodies which find use in these methods include antibodies to CD34, CD38, or other markers, which allow for selection of stem cells and removal of most mature cells.

Concomitantly or subsequent to the gross separation, a negative selection may be carried out, where antibodies to specific markers present on dedicated cells are employed. For the most part, these markers will include CD2, CD3, CD7, CD8, CD10, CD14, CD15, CD16, CD19, CD20 and glycophorin where combinations comprising at least CD3, CD8, CD10, CD19 and CD20, normally including at least CD10 and CD19 or at least CD2, CD14, CD15, CD16, CD19 and glycophorin are employed. These combinations of markers used to isolate hematopoietic T cell progenitor cells may vary as other markers become available. The hematopoietic cell composition substantially depleted of dedicated cells may then be further positively selected using a marker for Thy-1 (CD90), whereby a substantially homogeneous stem cell population is achieved. Exemplary of a progenitor cell population is a population which is $CD34^+$ $Thy\text{-}1^+$, which approximates the substantially homogeneous stem cell composition.

ISOLATION OF $CD34^+$ STEM CELLS

The $CD34^+$ molecule is a single chain type I transmembrane glycoprotein serving as a differentiation stage-specific receptor which is associated with hematopoietic T cell progenitor cells, stromal cell precursors, and microvascular endothelial cells. The $CD34^+$ stem cell population is composed of a heterogeneous mixture of cell types,

the major fraction represent committed progenitor cells and a minor fraction of $CD34^+$ stem cells remain capable of generating hematopoietic T cell progenitor cells in long term culture. There are a number of methods available for obtaining populations of $CD34^+$ cells. In one embodiment, bone marrow is used as the source for the $CD34^+$ cells. In another embodiment, $CD34^+$ cells are purified from peripheral blood mononuclear cells (PBMC).

CULTURE OF LYMPH NODE TISSUE FRAGMENTS

Coculture of hematopoietic T cell progenitor cells with lymph node cells results in an expansion in the number of cells and in the differentiation of cells into hematopoietic cells at all stages including mature T cells that are either $CD3^+CD4^+CD8^-$ or $CD3^+CD4^-CD8^+$.

In a normal adult mammal, T cells mature in the thymus, and then once mature, they emigrate out of the thymus to populate secondary lymphoid tissue, consisting of the lymph nodes (LNs) and lymphatic vessels. *In vivo*, LNs are normally found in locations such as the neck, axilla, abdomen, and groin, and are responsible for immune surveillance. A LN is an aggregation of lymphoid tissue (stroma) surrounded by a fibrous capsule that is found along the course of lymphatic vessels. The outer cortex contains populations of B lymphocytes within germinal center follicles, whereas the paracortical cords contain transiting T lymphocytes and antigen presenting cells (APCs). Some tissue from the two outer layers sends fibrous strands through the inner tissue layer, the medulla, to form sinuses which are lined with macrophages.

For the purposes of this disclosure, the term "LN material" comprises whole lymph nodes (LN), lymph node fragments (LNF), and lymph node cells. In one embodiment, LN material is obtained from a subject using standard surgical techniques well known in the art. The subject can be any mammal, preferably a primate, and more preferably a human. The LN material is used in an organ culture, such that the entire LN or a LNF is placed into a culture. In one embodiment, the LN material is placed directly into a liquid media on a substrate. A suitable substrate is laminin. However, any substrate can be used which plays a role in progenitor cell adhesion (e.g., via the β_7 integrins) or migration during early T cell differentiation.

Lymphoid cells can be derived from the disaggregation of a piece of lymph node. Alternatively, lymphoid cells can be derived from a LNF from which the lymph cells have migrated during tissue culture. Lymphoid cells migrating from LN material often form a monolayer of cells surrounding the isolated LN material. This phenomenon is known as "skirting". Lymphoid cells may include, but are not limited to, all cell types present in the lymph node which are not lymphocytes or lymphocytic precursors or progenitors. The lymph node cells are used to stimulate the T cell progenitor cells by coculturing the lymph node cells together with the T cell progenitor cells. In general, direct contact may be used for efficient stimulation. The lymph node cells may be obtained from the lymph nodes of a mammal at any time after the organ has developed to a stage at which it can support the differentiation of cells.

The lymph node cells can also be grown as a monolayer. A monolayer is an nonconfluent layer of lymph node cells, a confluent layer of lymph node cells having a thickness of a single cells, or a confluent layer of lymph node cells where the cells are stacked on one another to a thickness of two or more cells. The monolayer is formed of cells

disaggregated from their native tissue structure, which are then plated on a vessel and allowed to attach to a surface. In another embodiment, a lymph node cell line can be established and used to differentiate T cell progenitor cells. The cell line can be human, or derived from another mammalian species.

The LN material may be cryopreserved for storage and shipment to remote locations, such as for use in kits. Cells may be cryopreserved by suspending the cells in a cryopreservation medium and freezing the cell suspension. The cell suspension is generally frozen gradually and then stored in liquid nitrogen or at an equivalent temperature in a medium containing serum and a cryopreservative such as dimethyl sulfoxide (DMSO).

COCULTURE OF LYMPHOID CELLS AND CD34$^+$ PROGENITOR CELLS

Coculture of the CD34$^+$ progenitor cells with LN material results in the expansion and differentiation of the CD34$^+$ cells into T cells of various stages of maturity, including immature T cells that are (double positive) CD3$^+$CD4$^+$CD8$^+$ or single positive mature T cells that are either CD3$^+$CD4$^+$CD8$^-$ or CD3$^+$CD4$^-$CD8$^+$.

Various media are commercially available and can be used during the coculture process. Examples of suitable media include Dulbecco's Modified Eagle Medium (dMEM), Iscove s medium, and the like. Coculture media is frequently supplemented with serum, usually fetal bovine serum, generally at a concentration of from about 1-15%, preferably about 5%, vitamins, non-essential amino acids, and other cell culture reagents. For example, appropriate antibiotics to prevent bacterial growth and other additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM), 2-mercaptoethanol (1-10 x 10^{-5} M) may also be included. The pH of the media ranges from about 6.0 to 8.0, but is preferably 7.4 after 5% CO_2 equilibration. Typically, the osmolarity of the media ranges from 200 to 1000 mOsm, and in one embodiment is about 290-300 mOsm. The temperature of the media ranges from 30 to 40°C, and in one embodiment is 37°C.

Various devices exist for use with during the coculture process that allow for growth and maintenance of cells. These devices employ a number of mechanisms such as crossed threads, membranes, controlled medium flow, and the like to facilitate the growth of the cells, for removal of waste products, and replenishment of the various factors associated with cell growth. Conveniently, tissue culture plates or flasks can also be employed.

The cells may be cultured in the presence of a mitogenic agent. A mitogenic agent is an agent capable of supporting the expansion of a population of hematopoietic T cell progenitor cell descendants when incubated or cultured with these cells. These agents include, but are not limited to lectins, such as concavalin A and phytohemagglutinin.

In one embodiment, a cytokine is included during the coculture process. The term "cytokine" is used as a generic name for a diverse group of soluble proteins, peptides, and polypeptides, which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment.

Cytokines comprise interleukins, initially thought to be produced exclusively by leukocytes; lymphokines, initially thought to be produced exclusively by lymphocytes; monokines, initially thought to be produced exclusively by monocytes; interferons, initially thought to be involved in antiviral responses; colony stimulating factors, initially thought to support the growth of cells in semisolid media; and chemokines, thought to be involved in chemotaxis; and a variety of other proteins. Cytokines also comprise thymic hormones. Thymic hormones are postulated to promote T cell maturation. These hormones include thymosin, thymopoietin, thymulin, and thymic humoral factor. *In vivo*, the expression of most cytokines is strictly regulated; these factors are usually produced only by activated cells in response to an induction signal. Examples of other cytokines include: tumor necrosis factor, transforming growth factor-β, lymphotoxin, migration inhibition factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, various FLT receptor ligands such as flt 3, granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, and macrophage CSF (M-CSF).

In another embodiment, a combination of cytokines is included during the coculture process into combinations or cocktail formulations. Examples of the cocktails include: (1) IL-2; (2) IL-2 and IL-6 and oncostatin M (OSM); (3) IL-2, IL-6, IL-7, OSM, and stem cell factor (SCF); (4) IL-2, IL-6, IL-7, OSM, SCF, flt 3; (5) IL-2, IL-6, IL-7, OSM, SCF, flt 3, and thrombopoietin (TPO); (6) IL-2, IL-6, IL-7, SCF, flt 3, and TPO, and leukemia inhibitory factor (LIF); and (7) IL-2, IL-6, and LIF. Alternative combinations of these cocktails are also contemplated for use with the methods described herein.

The factors which are employed may be naturally occurring or synthetic, *e.g.*, prepared recombinantly, and may be human or of other species, *e.g.*, non-human primate or murine. In a preferred embodiment, a human cytokine is used. The amount of the cytokine factors will generally be in the range from about 0.1 ng/ml to 1000 ng/ml. Advantageously, a range from about 1 ng/ml to 100 ng/ml, and preferably from about 5 ng/ml to 50 ng/ml is used with the methods described herein.

ANALYSIS OF COCULTURED CELL POPULATIONS

A number of assay methods are contemplated to determine the differentiation state of the cocultured cell populations described herein. For example, a fluorescence-activated cell sorter (FACS) can be used to assay for mature T cells. For example, FACS is used in a flow cytometric analysis to monitor the developmental progress of CD34$^+$ cells through the various stages of T cell differentiation. The technique of FACS is well known in the art. Generally speaking, during a FACS assay, a population of cells is incubated with one or more probes that are each labeled with a different fluorescent tag. The different fluorescent tags each emitted a unique fluorescent signal that is used to differentiate the binding of one probe to a specific target on a cell. Using FACS analysis and probes for CD3, a marker for T cells generally, CD4, a marker for the T helper cell, and CD8, a maker for the T cytolytic cell, it is possible to monitor the differentiation of CD34$^+$ cells into T cells. It is also possible to assay various cytokines for their effectiveness as potential T cell differentiating cytokines.

In one aspect of this embodiment, lymph node mononuclear cells (LNMC), peripheral blood mononuclear cells (PBMC), stem cells (CD34$^+$ cells) are tested immediately following isolation, or as cocultures of LNMC and CD34+ stem cells. During the testing, cells are harvested from cultures at various time points during the study, stained using directly conjugated MAbs (Becton Dickinson, Franklin Lakes, NJ) and analyzed using a FACS Caliber (Becton Dickinson, Franklin Lakes, NJ) for expression of the following cell surface antigens: CD1A, CD3, CD4, CD5, CD8, CD10, CD11c, CD13, CD14, CD16, CD19, CD25, CD26, CD27, CD28, CD34, CD44, CD45RA, CD45RO, CD49f,d, CD51, CD56, CD57, CD62L, CD90, CD117, CD132, TCR ,TCR , HLA-DR, CCR5, CXCR4 or the appropriate immunoglobulin isotype controls. CMFDA labeled (CD34$^+$ cells) are also analyzed by flow cytometry.

Various immunological, histochemical, and molecular biological techniques may also be used to assay for maturing T cells. For example, a pTα assay using the polymerase chain reaction (PCR) can be used to determine the presence or absence of immature T cells. This PCR assay uses a reverse transcriptase polymerase chain reaction (RT-PCR) protocol to detect the presence of the alpha chain of the immature form of the T cell receptor (TCR), and thus the presence of maturing T cells. The technique of RT-PCR is well known in the art and is described in PCR Technology: Principles and Applications for DNA Amplification, Erlich, Ed., W.H. Freeman and Company, p. 91 (1992).

An additional method of assaying for the presence of newly differentiated T cells in the coculture milieu determines the presence of T cell receptor rearrangement excision circles (TREC) in a host cell. During the maturation process, excision circles composed of genetic material from the T cell receptor are generated in maturing T cells as the genetic components of the T cell receptor are rearranged into a mature form. The number of TREC molecules is highest in newly generated T cells that have recently undergone T cell receptor gene rearrangement. The number of TREC molecules in a cell decreases with each cell division. T cells that have few TREC molecules are likely to have undergone multiple cell divisions and may represent residual mature T cells within the LN material used in the coculture process as these cells are older than the population of T cells differentiated *de novo* from the progenitor cell population. Alternatively, T cells that contain relatively higher numbers of TREC are more likely to have differentiated from CD34$^+$ progenitor cells. Thus, an assay that can determine the number of TREC in a cell can be used to

determine the age of a T cell population. The TREC assay exploits PCR technology to amplify TREC sequences to determine the number of TREC in a target cell populations. A general TREC protocol is described in Douek DC, *et al.*, Nature 396(6712):690-5 (1998).

Another method for determining the presence of T cells which differentiated from CD34$^+$ cells involves the use of a retroviral vector as a marker or tag. Generally, a retroviral vector capable of integration into a host cell genome is introduced to the population of CD34$^+$ cells before coculture with the LN material. Methods of nucleic acid introduction into a target cell are discussed herein. The retroviral vector contains one or more markers which can be used to discriminate between CD3$^+$ cells that differentiated from a CD34$^+$ cell introduced during the coculture procedure, and any CD3$^+$ cells that might have been present in the LN material when it was taken from the host. The act of evaluating the cocultured population of T cells for the presence of previously matured CD3$^+$ cells can occur by screening for a marker or unique sequence contained within the retroviral vector using standard techniques well known in the art.

Other analytical tools are available with which to examine the cocultured cell population. For example, functional lymphocyte responses to recall antigens such as Candida and tetanus toxoid can be used in the lymphocyte proliferation assay (LPA) discussed below. Also, neoantigens can be introduced to the developing T cells during culture, the neoantigen can be used in the LPA to then examine the ability of the differentiated T cells to respond to novel, synthetic antigens. Also, recombination activation gene analysis is contemplated for use with the methods described herein.

Although specific methods of determining the presence of mature, differentiated T cells that originated from a population of CD34$^+$ stem cells are described herein, any method that permits the detection of mature T cells differentiated from a population of CD34$^+$ stem cells is contemplated for use with the methods described herein.

GENETIC MODIFICATION

The hematopoietic T cell progenitor cells or descendants thereof may be genetically modified by contacting the cells with a nucleic acid sequence capable of genetically modifying the hematopoietic T cell progenitor cells or descendants thereof. The resulting cells may then be grown under conditions as described for unmodified hematopoietic T cell progenitor cells, whereby the modified hematopoietic T cell progenitor cells may be expanded and differentiated and used for a variety of purposes. Genetic modification of a hematopoietic T cell progenitor cell includes all transient and stable changes of the cellular genetic material that are created by the exogenous addition of a nucleic acid sequence. Techniques for the modifying the progenitor cells by introducing nucleic acid sequences into CD34$^+$ stem cells are known to one of ordinary skill in the art (Dick *et al., Blood* 78:624-634, 1991; Rosenzweig, M., *et al., Blood* 90:4822-4831, 1997; Rosenzweig, M., *et al., J. Med. Primatol.* 25:192-200, 1997; Fletcher *et al., Blood* 76:1098-1103, 1990).

"A polynucleotide" or "a nucleic acid sequence" refers to a polymeric form of nucleotides at least 10 bases in length. By "isolated nucleic acid sequence" is meant a polynucleotide that is not immediately contiguous with both

of the coding sequence with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The nucleotides of use with the method of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term includes single and double stranded forms of DNA. In one embodiment, the nucleic acid sequence encodes a polypeptide of interest. The nucleic acid can also encode a mutagen, antisense, ribozyme, or a dominant negative form of a polypeptide of interest.

Nucleic acid sequences which encode a polypeptide of interest, or functional fragment thereof, can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the definition are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters, are included in the invention (see *e.g.*, Bitter *et al.*, *Methods in Enzymology* 153:516-544, 1987). Promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

The nucleic acid sequences employed can include a marker, which allows for selection of cells into which the DNA has been integrated, and against cells which have not integrated the DNA construct. Various markers exist, particularly antibiotic resistance markers, such as resistance to G418, hygromycin, and the like. Less conveniently, negative selection may be used, where the marker is the HSV *tk* gene, which will make the cells sensitive to agents, such as acyclovir and gancyclovir.

Also, multiple drug resistance gene(s), *e.g.*, *pgp*-1, may be introduced to protect the cells against cytotoxic drugs. In addition, nucleic acid sequences which provide resistance to viral infection or viral replication can be introduced. In one nonlimiting example, nucleic acid sequences which provide resistance to an immunodeficiency lentivirus infection such as HIV can be introduced. These nucleic acid sequences include antisense and ribozyme sequences. The transcription of ribozymes has been shown to protect against viral infection, or viral replication of an immunodeficiency lentivirus (Rosenzweig, M. *et al.*, *Blood* 90:4822-4831, 1997).

A number of nucleic acid sequences are contemplated for use with the present invention. A suitable nucleic acid sequence for introduction into a $CD34^+$ cell is a sequence whose expression or presence within the host $CD34^+$ cell results in an increase in the viability of the host cell. For example, when an antisense sequence that is complementary to the *tat/rev* Exon I is introduced into a cell infected with the HIV virus, the product of this nucleic acid sequence produces an short segment of antisense nucleic acid that interferes with the expression of both the *tat* and *rev* genes. The interference generated by the antisense sequences protects the host cell and other cells by limiting viral reproduction.

The nucleic acid sequences contemplated here can be prepared in a variety of conventional ways. Numerous vectors are now available which provide for the desired features, such as long terminal repeats, marker genes, and restriction sites. The vector can be introduced into an appropriate plasmid and manipulated by restriction, insertion of the desired gene, with appropriate transcriptional and translational initiation and termination regions, and then the plasmid is introduced into an appropriate packaging host. At each of the manipulations, the plasmid can be grown in an appropriate prokaryotic host, and then analyzed to ensure that the desired construct has been obtained. The construct can then be subject to further manipulation. When completed, the plasmid or excised virus can be introduced into the packaging host for packaging and isolation of virus particles for use in the genetic modification.

Many vectors such as viral vectors, episomal plasmid vectors, stably integrating plasmid vectors, and artificial chromosome vectors can be utilized with the methods of the invention. Viral vectors include retroviral vectors, adenoviral vectors, adeno-associated viral vectors, herpesviral vectors and pox viral vectors. In one embodiment, a retroviral vector is employed for the introduction of the DNA construct into the stem cell host. An example of a suitable retroviral vector is a lentivirus-derived vector. Vectors may be designed to integrate into a specific location in the genome by insertion or homologous recombination, may integrate randomly or may remain in the nucleus as a stable episomal nucleic acid.

Various retroviral vectors may be employed for genetic modification. Combinations of retroviruses and an appropriate packaging line, where the capsid proteins will be functional for infecting cells of a primate, such as a human, can be utilized. Various amphotropic virus-producing cell lines are known, such as PA12 (Miller *et al.*, *Mol. Cell. Biol.* 5:431-437, 1995), PA317 (Miller *et al.*, *Mol. Cell. Biol.* 6:2895-2902, 1986) GRIP (Danos *et al.*, *PNAS* 85:6460-6464, 1988). Usually, the cells and virus will be incubated for at least 24 hours in the culture medium. The cells are then allowed to grow in the culture medium for at least one week, and may be allowed to grow for two weeks or more, before analysis.

Other delivery techniques are well known in the art can be used to deliver a nucleic acid sequence capable of genetically modifying the hematopoietic T cell progenitor cells or descendants thereof. These techniques include, but are not limited to, a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles that are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley *et al.*, *Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino *et al.*, *Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the

targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

To prove that one has the modified hematopoietic T cell progenitor cells and descendants, various techniques may be employed. The genome of the cells may be restricted and used with or without amplification. The polymerase chain reaction, gel electrophoresis, restriction analysis, Southern, Northern, and Western blots may be employed, sequencing, or the like, may all be employed with advantage.

THERAPEUTIC INTERVENTION

Also provided here is a method of treating a subject having an immune disorder, by culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell to produce a descendent cell, and then administering a therapeutically effective amount of the T cell progenitor cell or a descendent thereof to the subject. By subject is meant any mammal, preferably a primate, more preferably a human. A therapeutically-effective dose of hematopoietic T cell progenitor cells refers to that amount of hematopoietic T cell progenitor cells or descendants thereof that is of sufficient quantity to alleviate a symptom of the disease or to ameliorate the immune disorder. "Ameliorate" refers to lessening or lowering the disease's or disorder's detrimental effect in the patient receiving the therapy. In the case of an immunodeficiency, (*e.g.*, AIDS), the treatment can increase the number of T cells.

There is also provided a method for expanding and differentiating cells *in vitro* which can then be used for therapeutic intervention in cases of immune disorders. Immune disorders include "immunodeficiency disorders" and " immunoproliferative" disorders, described above. An example of an immunodeficiency disorder is AIDS, and an example of an immunoproliferative disorder is a lymphoma. In this regard, a patient's own T cell progenitor cells can be removed, expanded and/or differentiated, and optionally genetically altered. The cells can then be reintroduced into the patient.

All of the transplantation treatments described can be used in conjunction with standard viral or anti-HIV treatments. In one embodiment, transplantation as described above would be accompanied by on-going antiviral treatments and more specifically anti-HIV-1 treatments.

Also described is a method for transplantation, typically of autologous cells, but also of syngeneic, allogeneic or xenogeneic cells. The hematopoietic T cell progenitor cells may be administered in any physiologically acceptable medium, normally intravascularly, although they may also be introduced into bone or other convenient site, where the cells may find an appropriate site for further differentiation or for activation. For example, the cells can be introduced into a lymph node or the spleen. Usually, at least $1x10^5$ cells will be administered, preferably $1x10^6$ or more. Transplantation or implantation is typically by simple injection through a hypodermic needle having a bore diameter sufficient to permit passage of a suspension of cells therethrough without damaging the cells. The cells may be introduced by injection through a hypodermic needle, catheter, or the like. If desired, depending upon the purpose of the introduction of the cells, factors may also be included, such as the cytokines IL-2, IL-6, leukemia inhibitory factor (LIF), IL-7, stem cell factor (SCF), flt 3, oncostatin M (OSM), and thrombopoietin (TPO), and others.

The introduction of nucleic acid sequences into the progenitor cells or descendants thereof can be used for a wide variety of purposes, such as gene therapy, introduction of novel capabilities into the hematopoietic T cell progenitor cells, virus resistance, enhancement of maturation to a particular subset, or the like. There are many genetic diseases specific for hematopoietic cells, including combined immunodeficiency and leukemia. These diseases may be treated by homologous recombination, where at least one copy of the defective gene may be modified to the wild-type or a functioning gene. It may be necessary to correct only one copy to sufficiently provide for therapeutic treatment of such disorders. There are numerous descriptions of methods of homologous recombination in the literature (see for example, Mansour *et al. Nature* 336:348-352, 1988, and Schwartzberg *et al., PNAS USA* 87:3210-3214, 1990).

Alternatively, one may use the hematopoietic T cell progenitor cells and their progeny as carriers for the production of a wide variety of products, where the host is genetically deficient or as a result of a subsequent disease has become genetically deficient. Genetic diseases involving lack of a particular natural product include muscular dystrophy, where there is a lack of dystrophin, cystic fibrosis, Alzheimer's disease, Gaucher disease, etc. In those instances where a particular polymorphic region of a polymorphic protein such as a T-cell receptor or major histocompatibility complex antigen which is involved with susceptibility to a particular disease, *e.g.*, an autoimmune disease, the particular exon may be "knocked out" by homologous recombination. In this manner hematopoietic cells are provided which will not be susceptible or responsive to the disease process.

In many situations the therapy involves removal of bone marrow or another source of hematopoietic T cell progenitor cells from a human host, isolating the hematopoietic T cell progenitor cells from the source and expanding and differentiating the cells. The host may be left intact, or may be treated to substantially or complete ablate native hematopoietic capability. The hematopoietic T cell progenitor cells may be modified so as to provide for hematopoietic T cell progenitor cells having the desired genetic modification. After completion of the treatment of the host, the modified hematopoietic T cell progenitor cells or descendants thereof are then restored to the host to provide for the new capability. If necessary, the process may be repeated to ensure the substantial absence of the original hematopoietic T cell progenitor cells and the substantial population of the modified hematopoietic T cell progenitor cells and descendants thereof.

In another embodiment, lymph node tissue can be conditioned *in vivo* to induce the proliferation or differentiation of progenitor cells by the administration of cytokines. Examples of the cocktails include: (1) IL-2; (2) IL-2, IL-6 and OSM; (3) IL-2, IL-6, IL-7, OSM, and SCF; (4) IL-2, IL-6, IL-7, OSM, SCF, flt 3; (5) IL-2, IL-6, IL-7, OSM, SCF, flt 3, and TPO; (6) IL-2, IL-6, IL-7, OSM, SCF, flt 3, TPO, and LIF; and (7) IL-2, IL-6, and LIF. Alternative combinations of these cocktails are also contemplated for use with the methods described herein.

The cytokine compositions described above are preferably prepared and administered in dose units. "Administering" the compositions may be accomplished by any means known to the skilled artisan. Solid dose units are tablets, capsules and suppositories. For treatment of a subject, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patent, different doses are necessary.

Under certain circumstances, however, higher or lower doses may be appropriate. The administration of the dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The cytokine compositions may be administered locally or systemically. For local administration, the compositions can be administered by injection. For systemic administration, the compositions according to the invention are preferably administered intravenously. However, other routes of administration are within the scope of the invention. Thus, the compositions can be administered topically, intravenously, orally or parenterally or as implants, but even rectal use is possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro) capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science*, 249: 1527-1533, 1990.

By "therapeutically effective dose" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest the symptoms of the disease and its complications. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the patient. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Gilman *et al.* (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, PA.

METHOD FOR THE IDENTIFICATION OF AGENTS THAT AFFECT T CELL DIFFERENTIATION AND/OR GROWTH

In another aspect of the invention, a method for testing the effect of an agent on cells is provided. The term "agent" as used herein describes any molecule, *e.g.*, a protein, peptide, pharmaceutical or biological agent with the capability of altering growth or differentiation of hematopoietic T cell progenitor cells. The agent can be anything known to be or suspected of being capable of affecting the growth and development of T cells. These agents include synthetic chemical agents, biochemical agents, cells, extracts, and homogenates. The test agent may also be a combinatorial library for screening a plurality of compounds. Compounds identified can be further evaluated, detected, cloned, sequenced, and the like, either in solution of after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence, such as PCR, oligomer restriction (Saiki et al., *Bio/Technology* 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner et al., *Proc. Natl. Acad. Sci. USA* 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren *et al.*, *Science* 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren *et al.*, *Science* 242:229-237, 1988).

Candidate agents for use with the present method encompass numerous chemical classes. They can be organic molecules, preferably small organic agents having a molecular weight of more than 50 and less than about 2,500 Daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural agents. For example, numerous means are available for random and directed synthesis of a wide variety of organic agents and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural agents in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and agents are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

The coculture of the lymph node cells and hematopoietic T cell progenitor cells can be carried out in the presence of the agent. "Incubating" includes conditions which allow contact between the test compound and the hematopoietic T cell progenitor cells and descendants thereof. "Contacting" includes in solution and solid phase. The growth and/or differentiation of the progenitor cells, or descendants thereof, contacted with the agent is then compared to the growth or differentiation of control progenitor cells or descendants thereof as a determination of the effect of the agent. In one embodiment, the control cells can be a control culture subject to otherwise identical conditions, but without the agent. Other suitable controls can readily be determined by one of skill in the art. For example, the control cells can be incubated with a vehicle, or can be incubated with a different dose of the agent, or can be incubated with an agent of known effect and activity. A time course, wherein the cells are exposed to an agent for varying amounts of time, can also be performed. Similarly, a study wherein cells are exposed to different concentrations of an agent, can be performed.

KITS

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means containing one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. The kit includes a cryopreserved sample of lymph node or a cryopreserved sample of a suspension of lymph node cells, together with instructions for co-culturing such cells with hematopoietic T cell progenitor cells. The instructions may be specific to primate cells, such as human cells. The kit may also contain a container comprising one or more cytokines, or a combination of

cytokines. For example, a cytokine combination comprises at least IL-2 and IL-6. The cytokine combination may further comprise leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, thrombopoietin (TPO), oncostatin M (OSM), or IL-7. More than one container containing different cytokines, or cytokine combinations, can be included in the kit.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following examples are illustrative only, and not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLES

Example 1

Method of Isolating CD34$^+$ Cells from Bone Marrow

Bone marrow is one of the richest source of CD34$^+$ cells (1-3% in normal adult individuals). U.S. Patent No. 5,766,944, describes a method for purification of CD34$^+$ cells from bone marrow. Briefly, bone marrow aspirates were obtained from a human source using standard techniques well known in the art, and diluted 1:2 with sterile PBS or normal saline and mixed gently. Depletion of red blood cell/high density cells was accomplished by the addition of an equal volume of 3% gelatin that was mixed gently and allowed to settle by gravity sedimentation. Sedimentation time may vary with different donors and the quality of the marrow aspirate. When the red blood cell fraction occupied 20% of the initial volume and there was a definite interface between the leukocyte rich plasma layer and the sedimented red blood cell (SRBC) layer, the nuclear cells were harvested by pipetting the plasma layer into 50 ml tubes. Cells were washed with NS or PBS and centrifuged at 1350 rpm.

The RBC depleted fraction was resuspended in 35 ml of complete media (such as Dulbecco's modified Eagle's medium (DMEM)(GIBCO, BRL, Grand Island, N.Y.), RPMI-10, or HAM's F12$^+$ supplements, to approximate the 1 to 3.5 dilution of whole blood for ficoll-hypaque, which was then underlaid with 15 ml of ficoll-hypaque (d=1.077 g/ml) for low density cell centrifugation. The ficoll-hypaque gradient was then centrifuged at 1350 rpm (400 *g*) at room temperature (22°C) for 35 minutes. Then, the interface mononuclear cells were harvested with a pipette. The mononuclear cells were then diluted with an equal volume of PBS, spun at 1000 rpm for 10 minutes, and the supernatant was aspirated and discarded. The cells were resuspended in PBS +2% FCS, spun and aspirated two more times. The mononuclear marrow cells were resuspended in 5 ml RPMI and counted. The mononuclear marrow cell concentration was then adjusted to 10×10^6 cells/ ml with RPMI-5% HI-ADS-FCS (note-maximum of 70×10^6 cells/tube).

A 0.14M 2-aminoethylisothiouronium bromide (AET) solution was prepared and mixed 4:1 with sedimented red blood cells (SRBC) and incubated at 37°C for twenty minutes. The AET treated SRBC's (AET-SRBC) was then washed four times with PBS. A 1% AET-SRBC solution was prepared by diluting 0.5 ml of packed AET-SRBC with 50 ml PBS. An equal volume of the 1% AET-SRBC solution was then added to the mononuclear marrow cell suspension and the mixture was incubated at 37°C for 5 minutes. The cell mixture was then centrifuged at 600 rpm for 5 minutes then incubated at 4°C for 60 minutes. Alternately, a 2% AET-SRBC solution mixed with a cell suspension of

2.5 x 10^6 cells/ml was used to deplete CD3$^+$ T cells. A sample of the rosetting cells was checked under the microscope as rapid rosetting with large agglutination to entrap non-T cells, the percent rosetting should not exceed that of a t_o sample.

After the one hour incubation, the cells were gently resuspended so as not to shear the CD2/CD58 complex between T cells/SRBC. The cell suspension was gently underlaid with a volume of ficoll-hypaque which was 2 times the cell suspension volume. The ficoll-hypaque gradient was centrifuged at 600 rpm for 10 minutes and then at 1350 rpm for 25 minutes. The T cells were located in the pellet and the non-T cells (B cells, macrophages/dendritic cells, mixed stem cell population including CD34$^+$ cells) were located at the interface. The interface mononuclear cells was harvested with a pipette. The cells were diluted with an equal volume of PBS, centrifuged at 1000 rpm for 10 minutes, aspirated and the supernatant was discarded. The wash step was repeated.

The cells were then resuspended in complete RPMI (without serum) and the cell concentration was adjusted to 2 x 10^6 cells/ml. Non-CD34 cells were then depleted by immunomagnetic selection.

The cells were resuspended in 2-3 ml complete media and a cell count was performed. The CD34$^+$ cell recovery was calculated. An aliquot was reserved for Colony Forming Unit Assays/phenotyping in a separate tube with complete media. The CD34$^+$ cells typically display sheared epitopes and/or down regulation of the receptor induced by the binding of the capture antibody. The CD34$^+$ receptor recycles within 24-36 hours after binding to the capture antibody in the selection process. The aliquot reserved for phenotyping should therefore be rested prior to testing. There may be considerable variability in detection of the CD34 receptor among different mAb α-CD34 used for FACS analysis. The epitope which has provided an even performance is the HPCA-2 (8G12 clone).

The CD34$^+$ cells were now ready to be used. If the CD34$^+$ cells are to be co-cultivated with lymphoid cells, the absolute minimal stem cell concentration should be about 10,000 cells per fragment/monolayer. The cells will undergo a rapid expansion during the initial phase of the coculture reflecting the expansion and terminal differentiation of the pre-committed progenitors in the CD34$^+$ population. At set intervals of the coculture, prior to functional testing or phenotyping these "non-mononuclear" cells were removed after cell harvesting by low density cell centrifugation with ficoll-hypaque.

Example 2

Method of Isolating CD34$^+$ Cells from Peripheral Blood

The peripheral blood mononuclear cells (PBMCs) were obtained by ficoll-hypaque (F/H) centrifugation as described above. PBMC were resuspended in RPMI 1640 + 5% heat inactivated FCS+ 100 U/ml DNase (100 U/ml; BM) at a cell concentration of 1-2x10^7/ml and were separated in CD3$^+$ and CD3$^-$ cell populations by standard AET-sheep red blood cell (sRBC) rosetting. In order to obtain mature CD3+ T cells to use as controls, SRBC-CD3$^+$ cells were depleted of sRBC by treatment with ACK RBC lysing buffer and cultured on laminin coated plates in media containing one of seven cytokine cocktails (#1-7) with or without INDINAVIR (CRIXIVAN) (7 μM) (Merck, Whitehouse Station, NJ).

The cocktails were:

(1) IL-2; (2) IL-2, IL-6, and OSM; (3) IL-2, IL-6, IL-7, OSM, and SCF; (4) IL-2, IL-6, IL-7, OSM, SCF, and flt 3; (5) IL-2, IL-6, IL-7, OSM, SCF, flt 3, and TPO; (6) IL-2, IL-6, IL-7, SCF, flt 3, TPO, and LIF; (7) IL-2, IL-6, and LIF.

The remaining T and NK cell-depleted population was further depleted of monocyte/macrophages with 20-30 minutes of plastic adherence. The non-adherent population was then selected for $CD34^+$ cells using immunomagnetic anti-CD34 Mab and/or anti-AC133 Mab coupled beads. An aliquot of $CD34/AC133^+$ cells was analyzed by flow cytometry for purity and the population was reselected if more than 1% $CD3^+$ cells were present. $CD34/AC133^+$ cells were added to culture wells, as well as cultured alone in 96 RB laminin coated wells at 5,000 cells/well in various cytokine containing media. In certain experiments, the $CD34/AC133^+$ cells were tagged with the fluorescent marker 5-chloromethylfluorescein diacetate (CMFDA)(Molecular Probes, Eugene, OR) as per manufacturer instructions prior to adding to lymph node fragment containing wells. Several LNF wells treated with the various cytokine combinations were not pulsed with $CD34/AC133^+$ cells to serve as CD34+ progenitor cell controls.

Example 3

Method of Isolating Lymph Node Material

In a specific aspect of the above-described embodiment, inguinal LNs were removed according to standard NIH/NIAID protocols (Protocol No. #92-I-0125) from both HIV uninfected and HIV-infected individuals under local anesthesia. One to two (1-2) mm sections were cut from the LN for pathologic and HIV RNA *in situ* analyses prior to removal of the fibrous capsule, fat and scar tissue from the remainder of the tissue. The tissue was then cut into consecutively smaller pieces allowing a portion of the lymph node mononuclear cells to be harvested to serve as controls, to migrate out into the media over time. The final LNFs (1-2 mm) were then placed (1-6 LNFs/well) on laminin (30 μg/ml; Gibco/Life Science Products) coated wells (24 or 12 well plates) in 75-200 μL of media, covered with sterile glass cover slips and incubated at 37°C for 1 hr. Cytokine-containing media (in some cases ± INDINAVIR; 7 μM) were added to cover the LNFs. Cultures were incubated for 1 week without media changes and observed for monolayer skirting of lymphoid cell components around the LNF. Electron microscopy was used to examine the extent of skirting and the degree of cellular migration from the LNF. Cultures were then fed twice a week with 50% volume replacement of cytokine- containing media ± INDINAVIR thereafter. In the case of LNFs obtained from HIV-infected subjects, the culture supernatant was saved and stored at -80°C for later analyses of HIV p24-content.

Example 4

Culture Method

Cell populations were maintained in a base media [1:1 vol/vol Iscoves modified essential media: HAMS F12 media plus 100 U/ml penicillin/streptomycin, 1mM glutamine, 1 mM hepes buffer, 1% sodium pyruvate (all from Gibco/Life Science Products), 1% BIT (Stem Cell Technologies, Vancouver, Canada), 0.5% MEM vitamins (Sigma), 0.5% non-essential amino acids (Sigma), 0.5% linoleic acid (Sigma) and 5% fetal bovine serum containing one or more of the following cytokines (Peprotech, Rocky Hill, NJ) with or without the addition of 7 μM INDINAVIR (Merck): a) Interleukin (IL)-2 at 20 U/ml, b) IL-6 at 50 ng/ml, c) IL-7 at 10 ng/ml, d) Oncostatin M (OSM) at 10 ng/ml, e) stem cell

factor (SCF) at 100 ng/ml, f) flt 3 at 100 ng/ml, g) thrombopoietin (TPO) at 50 ng/ml and h) leukemia inhibitory factor (LIF) at 10 ng/ml. The following seven cytokine combinations were tested for their ability to support lymphopoiesis in LNFs: #1 (a) only; #2 (a+b+d); #3 (a-e); #4 (a-f); #5 (a-g); #6 (a-c plus e-h) and #7 (a+b+h).

Example 5

Coculturing LN Material and CD34$^+$ Cells

The LN material was placed in the bottom of the upper chamber of a transwell plate containing Iscove's/Ham's medium at a 1:1 ratio supplemented with 5% FCS, 0.4 µg/ml hydrocortisone (Calbiochem Behrig, La Jolla, CA), 11 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), 1 x 10^{-10} M cholera enterotoxin (Sigma), 5 µg/ml insulin (Sigma), 1.8 x 10^{-4} g/ml sodium pyruvate, and 50 µg/ml gentamycin.

The media was changed every 4-5 days until about 48 hours prior to the addition of stem cells. Prior to initiating the coculture phase by the addition of purified stem cells, the lymphoid culture media was mixed 1:1 with the coculture type media in order to wean the lymphoid fragments to the lymphocyte type coculture media. (Coculture Media: Iscove's/Ham's medium at a 1:1 ratio containing 5% FCS, 10% HL-1 serum free media (Ventrex, Portland, ME), 11 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), and 25 U/ml of IL-2 at 37°C in a 5% CO_2 atmosphere).

At the time the purified CD34$^+$ stem cells were ready to be added to the LN material, the lymphoid culture media was completely aspirated from the upper and lower chambers of the transwell and coculture media was added to the capacity of the outer well and to the halfway point of the inner well. The stem cells were then seeded to the upper well of the transwells containing the lymphoid fragments at a concentration of between 10,000 and 100,000 CD34$^+$CD38$^-$ stem cells per fragment (depending on the lineage status).

The lymphoid fragments which are being cocultured with the purified stem cells were checked periodically under the microscope for CD34$^+$ cell expansion (to precommitted lines) and for assessing whether a portion of the cells were tracking to the lymphoid fragments.

Approximately 10-20 days following initial culturing of the LN material, autologous CD34$^+$ stem cells were added to LN material containing wells (10,000-70,000/well) and monitored by a variety of parameters for indicators of lymphopoiesis. In certain cases, CD34$^+$ stem cells were added to LNF containing wells several times over approximately a 1 month period. The lymph node mononuclear cells harvested at the time of initial LN fragmentation, and PBL, obtained at the time of CD34$^+$ stem cell isolation, were cultured in identical cytokine/growth conditions in parallel at approximately 10^6/ml on laminin coated 6 well plates.

Example 6

A Polymerase Chain Reaction pTα Assay

A pTα assay using a reverse transcriptase polymerase chain reaction (RT-PCR) assay was used to determine the presence or absence of immature T cells. The RT-PCR assay involved the use of the reverse transcriptase enzyme which catalyzes the polymerization of deoxyribonucleic acid (DNA) using a ribonucleic acid (RNA) template. The RNA used in the RT-PCR method was isolated from a sample of cells taken from the coculture using TRIAZOL (Life

Technologies, Rockville, MD). The cells were pelleted by centrifugation and resuspended in 1 ml of TRIAZOL. To the suspension was added 200 µl of chloroform. This solution was then centrifuged at 14,000 rpm for 30 minute. When the centrifugation was complete, the aqueous layer was removed from the tube containing the sample. Following removal of the aqueous layer, 2 µl of Glycoblue (Ambion, Austin, TX) was added to the tube. Following this addition, 400 µl of isopropyl alcohol was added to the solution. This mixture was then centrifuged at 14,000 rpm for 10 minutes. Following completion of the centrifugation step, the resulting pellet was washed twice with 70% ethanol and then air dried for 10 minutes.

The yield of the above-described method was a nucleic acid pellet derived from the cultured cells. The next step of the assay method was to quantitate the amount of RNA present in the pellet and to determine the purity of the yield using ultraviolet spectroscopy using an absorbance ratio of Å260/280. This ratio yields information on the nucleic acid to protein absorbance present in the sample. Following this analysis, the nucleic acid sample was treated with 3U DNase for 30 minutes at 37°C to digest any deoxyribonucleic acid that was present in the sample.

Once the sample was purified, a complementary DNA strand was synthesized from the isolated RNA. The cDNA was constructed using the reverse transcriptase protocol from the SUPERSCRIPT KIT (Life Technologies, Rockville, MD). According to this protocol, in a 20 µL reaction volume was placed: the purified RNA and 100 Units of Moloney Murine Leukemia Virus Reverse Transcriptase. The solution was brought to a final concentration of the following compounds: 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, and 500 µM of the four deoxyribonucleosides (dNTPs).

Once the cDNA synthesis was complete, the resulting product was used as a template for the polymerase chain reaction. The primers used in the reaction were: Forward Primer Sequence: 5'-GGCACACCCTTTCCTTCTCTG-3' (SEQ ID NO. 1) and Reverse Primer Sequence: 5'-GCAGGTCCTGGCTGTAGAAGC-3' (SEQ ID NO. 2).

The PCR conditions were as follows: an initial heating step at 94°C for 2 minutes followed by 56°C for 2 minutes. Next, the thermocycler in which the PCR samples were inserted were processed for 35 cycles. The cycles consisted of the following steps: first, a round of heating for 30 seconds at 94°C, then a 30 second heating round at 56°C, and then a heating round for 1 minute at 72°C.

Following the completion of the PCR protocol, the PCR amplicons were run on a 2% agarose gel to examine the extent of pT alpha amplification. The amplicon from pT alpha was approximately 620 base pairs and compared in intensity with controls subjected to the same PCR conditions. The controls were RNA from fetal thymic tissue, PBMC, $CD4^+$ T cells, and $CD34^+$ stem cells, and an immature T cell line (MOLT-4) known to express pT alpha.

Example 7

A T cell Receptor Excision Circle Assay

A TREC assay for use with the method described herein includes the following steps and materials. TREC standards were prepared from a plasmid containing an insert that corresponded to the signal joint (sj) region of TCR delta. Standards were prepared at 10 fold dilutions that ranged from down 10^2 to 10^7.

Cells to be assayed in the TREC system were isolated from the coculture, pelleted, and frozen. Frozen cell pellets were dissolved in proteinase K in a 10mM Tris-HCl pH 8.0 solution, at a concentration of 100,000 cells per 10 µl. Cell lysates were incubated for 2 hours at 56°C to digest protein. The proteinase K was then inactivated by heating the cell lysate sample at 94°C for 15 minutes. Samples were then stored at -20°C until assayed.

To conduct the PCR assay 5 µl of cell lysate (50,000 cells) was added to 20 µl of master PCR mix. The master mix consisted of 2.5 µl of 10X PCR Buffer; 1.75 µl 50mM $MgCl_2$; 0.5 µl 10mM dNTP; 1µl 12.5µM 5' sj primer; 1µl 12.5µM 3' sj primer; 1µl 3.75µM probe; 0.25µl 5U/µl Taq; 0.125µl of BD636 reference; and, 11.875µl of water for a total volume of 20 µl.

Once prepared the samples were amplified on a Perkin Elmer (PE) (Norwalk, CT) PE 7700 SDS for 40 cycles of 95°C for 5 minutes followed by 60°C for 1 minute. Data from patient samples was compared to the standards calculated from the same run. TREC were calculated per 100,000 cells.

Example 8

Lymph Node Cells Support T Cell Differentiation

This Example shows that lymph node cells isolated from a subject can be used to generate differentiated T Cells. A chronically infected HIV$^+$ patient with degenerative changes in lymph node tissue was examined for the ability to assist in the generation of mature T lymphocytes from T lymphocyte precursor cells. Assessment of HIV viral load was performed by ELISA, in which the p24 antigen was measured using the commercially available HIV-1 p24 Antigen Assay (Coulter Corporation, Miami, FL). Subject R021898 had CD4 count of < 50, a high viral load, and was insensitive to INDINAVIR. An HIV uninfected individual was used as a control.

In this Example the procedure used to generate the mature T lymphocytes was as follows. LNF fragments were isolated and cocultured with CD34$^+$ T cell progenitor cells as described above. Additionally, several different controls were generated for use in the analysis. Cellular growth of the LN material alone, the CD34$^+$ cells alone, and the coculture of lymph node material plus CD34+ progenitor cells were monitored for viability using standard cell culture techniques, such as thymidine blue dye analysis.

The coculture of LNF fragments and CD34$^+$ progenitor cells was conducted *in vitro* under seven different T cell differentiating cytokine cocktails either in the presence or absence of INDINAVIR. The cocktails were:

(1) IL-2; (2) IL-2, IL-6, and OSM; (3) IL-2, IL-6, IL-7, OSM, and SCF; (4) IL-2, IL-6, IL-7, OSM, SCF, and flt 3; (5) IL-2, IL-6, IL-7, OSM, SCF, flt 3, and TPO; (6) IL-2, IL-6, IL-7, SCF, flt 3, TPO, and LIF; (7) IL-2, IL-6, and LIF.

The lymphoproliferative assays (LPAs) were performed on LN material and peripheral blood samples immediately following isolation and, in parallel with CD34$^+$ cell/LN material cocultured cells, at 1-2 time points following the addition of CD34$^+$ cells to LN material (5-10 weeks). Briefly, 10^5 cells were cultured (RPMI 1640 plus antibiotics, hepes buffer, glutamine and 20% human AB serum) in triplicate in a 96 well round bottom plate with or without the addition of 10% autologous antigen presenting cells (APC; EBV-transformed B cell lines or mitomycin C

treated T cell depleted PBMC) and one of the following stimuli: no stimulus, keyhole limpet hemagglutinin (KLH; 1 μg/ml), phytohemagglutinin (PHA) (10 μg/ml, Sigma), *Staphylococcus aureus* enterotoxin (SE-A) or (SE-B) at 10 μg/ml (Sigma), allo-antigen (pool of allogeneic EBV-transformed B cell lines), tetanus toxoid (TT) (37 U/ml), Candida (5 μg/ml) or HIV p24 antigen (0.5 μg/ml, (ABI, Columbia, MD)). Cultures were pulsed with ^{3}H thymidine (0.5 μCi/well (Dupont/NEN, Boston, MA)) either at 3 days (PHA, untreated), at 5 days for allo-antigen, *Staphylococcus aureus* Enterotoxin-A (SE-A) and *Staphylococcus aureus* Enterotoxin-B (SE-B), or at 7 days for KLH and untreated control or (TT, Candida, p24) following exposure to stimuli. Cultures were incubated with thymidine for 8-16 hours, cells were harvested and counted for thymidine incorporation.

Cells were harvested at serial intervals and examined for the presence of T cell markers using FACS analysis. Serial FACS analysis of the cultured cells was performed to analyze the stages of T cell differentiation from the $CD34^+$ progenitor cells and to determine the presence or absence of contaminating T cells that may have been co-purified with the LN material.

FIGURE 2 shows a number of FACS results obtained from the cells of Subject RO21898 that were grown using the culture methods described herein. FIGURE 2 graphs A and B show the presence of $CD3^+CD8^+$ (56.5%) and $CD3^+CD4^+$ (9.1%), respectively which were obtained from PBMCs on day #1. FIGURE 2 graphs C and D examined cells taken from the isolated LN material which showed $CD3^+CD8^+$ (0.5%) and $CD3^+CD4^+$ (0.1%), respectively. These results show that although there were $CD3^+CD8^+$ and $CD3^+CD4^+$ single positive cells present within the subject's immune system, these cells were substantially removed from the isolated LN material.

FIGURE 2 graph E examined the expression of $CD4^+$ vs. $CD8^+$ cells from the LN material on day 14, just prior to the addition of the $CD34^+$ cells, in the presence of T cell cocktail No. 1. The cocktail used here was a control to detect the presence or absence of contaminating T cells in the isolated LN material. In graph E, those cells that displayed both the CD4 and CD8 markers appeared in the upper right quadrant of the graph (Stage 1, 2, and 3 of developing double positive T cells). This quadrant in graph E shows only a population of 2.2% cells with these markers. Graph F, shows FACS results looking at the expression of CD3 and CD4 markers. This graph shows a reading of 8.9% for cells displaying both markers (single positive mature CD3+CD4+ T cells).

FIGURE 2, graphs G and H show results from the culture that was grown in T cell differentiating cocktail No. 4. Graph G shows only a small percentage of cells in the LN material were $CD4^+CD8^+$ double positive cells on day #14. Graph H shows that 8.0% of the cells examined were $CD3^+CD4^+$ cells (single positive mature $CD3^+CD4^+$ T cells). The single positive mature $CD3^+CD4^+$ T cell results were similar between cocktails 1 and 4 which suggest that mature T cells present in the LN material expand in a similar fashion under the influence of these cytokines.

After the measurements in graphs E-H were made, the $CD34^+$ cells were added to the LN material culture as described above. The remaining graphs show various results obtained from the cocultured cells grown in the presence of T cell differentiating cocktails Nos. 1 and 4. The results in graphs I and J are of particular importance. In graph I, which examined the dual expression of $CD4^+CD8^+$ cells, the upper right quadrant where such cells would appear, was nearly vacant. This results indicates that only a very small number of cells bearing CD4 or CD8 epitopes were present

in the LN culture that responded to T cell differentiating cytokine cocktail No. 1. The cytokines present in cocktail No. 1 are capable of promoting the expansion of $CD3^+$ cells that might have been contaminating the starting materials. However, from these results it is clear that few, if any, of these cells survived the LN purification process.

In contrast to the results of graphs I and J are the results of graphs K, L, and M. On day 45, $CD34^+$ cells grown in the presence of LN material and T cell differentiating cocktail No. 4 showed a large number (58%) of $CD4^+CD8^+$ double positive cells. Also, substantial populations of $CD4^+$ or $CD8^+$ cells (mature T cells) were also present. The displayed results of graph K are typical of developing thymocyte population obtained from a healthy subject. Thus, the results discussed show that the methods described herein are capable of producing a viable population of T cells expressing both CD4 and CD8 markers.

The results shown in FIGURE 3 demonstrate the complex expression of CD3, CD4 and CD8 receptors which occur during T cell differentiation. The level of expression of CD8 increased along the y-axis, the level of expression of CD4 increased along the x-axis and the level of expression of CD3 increased along the z-axis (oriented toward the viewer). The 3-D cube on the left represents $CD34^+$ progenitor cells cocultured with LN stroma in cytokine combination #1, there were only stage 4 single positive mature T cells present, single positive $CD3^+CD8^+$ T cells are represented along the rear left plane and single positive $CD3^+CD4^+$ T cells are represented along the forward plane. This is in contrast to the 3-D cube on the right which represents $CD34^+$ progenitor cells cocultured with LN material in cytokine cocktail #4. A new population of double positive $CD4^+CD8^+$ are evident which are expressing variable levels of CD3 (stages 1, 2, and 3). There were transitional stage 4 single positive mature T cells present along the base of the double positive population along with mature single positive $CD3^+CD8^+$ T cells which are represented along the rear left plane and mature single positive $CD3^+CD4^+$ T cells which are represented along the forward plane.

The results shown in FIGURE 4 are the Stimulation Indexes of various samples stimulated with PHA. PHA is a mitogen which stimulates cellular replication in lymphocytes. The Stimulation Index is an adjusted presentation of the number of radioactive counts found in a particular cell population over a control. Specifically, the Stimulation Index is the number of counts produced in an experimental well divided by the number of counts in a corresponding negative control well. Thus the Stimulation Index represents the number of counts in a particular well expressed in the number of fold above or below the unstimulated control. Typically, a three-fold increase in counts from an experimental well is required for the incorporation of thymidine to be considered relevant.

In FIGURE 4, the first column (Control), represents lymphocytes purified from an HIV negative donor. The remaining columns show results from various samples taken from subject RO21898. The second column (LNMC d-1) shows the Stimulation Index results from LN material taken from day 1, which indicate there was only limited incorporation of the label. The remaining columns indicate the Stimulation Index measurements taken on day 80 for cocultured cells with T cell differentiating cocktails Nos. 1 through 6. In the third column (LN d80#1), cells cultured in the presence of cocktail No. 1 produced cells that responded to PHA stimulation, but only responded nominally compared to Day#1 LNMCs. A similar pattern of stimulation is seen in the fourth column (LN d80#2), however, the magnitude of stimulation was significantly higher for the cells stimulated with PHA (simulation index = 26). The

stimulation pattern in the fifth column (LN d80#3) was approximately (14) on the Stimulation Index, the responses for the cells cultured in the presence of cocktail No. 3 were about equal in magnitude to cocktail No. 1.

The results shown in the sixth column (LN d80#4) showed the highest stimulation response for all of the culture conditions. These cells produced a Stimulation Index reading of (25) when stimulated with PHA.

The results from the seventh (LN d80#5) and eighth (LN d80#6) columns were less pronounced but still well above the LNMC d#1 levels of approximately (3). The seventh column had measurements of (10) for PHA stimulation. The eighth column also had a (10) for PHA stimulation.

The results shown in FIGURE 4 support a number of observations. First, as compared to the HIV negative control, the lymphocyte proliferative response of cells obtained from the peripheral blood of the test subject was reduced (data not shown), indicating a need for lymphocyte replenishment. The results indicate that the LN material isolated from the subject had little lymphocyte proliferative capabilities, indicating little contaminating lymphocyte material, even though the LN material was not completely T lymphocyte depleted. This result is supported by the results in the column labeled LN d80#1. $CD34^+$ cells cocultured in the presence of these cytokines were not expected to differentiate into mature T cells. Instead, this combination of cytokines was expected to stimulate only those cells that had already been mature T cells. So, any proliferation or stimulation observed in the LN d80#1 column was likely to have been caused by lymphocytes contaminating either the $CD34^+$ or the LN material (i.e., expanded mature T cells). Contamination appeared to be limited since the Stimulation Index readings for this column were only elevated when these cells were exposed to PHA.

In examining the effectiveness of the remaining T cell differentiating cytokine cocktails, the results show that each of the cocktails was capable of stimulating T cell differentiation from the $CD34^+$ cell populations cocultured with the LN material. Cocktail No. 2 appeared to stimulate cells that were relatively sensitive to PHA stimulation. Cocktails Nos. 2 and 4 showed the highest increase in stimulation as compared to the LNMC d-1 and LN d80#1 controls. Cocktail No. 5 and 6 did not produce stimulation results higher than those seen with cocktail No. 1 when the cells were stimulated with PHA, but Cocktail No. 5 did produce higher stimulation results than seen in LNd80#1 when the cells were stimulated with the allo-antigen (see Figure 5). These results show that all of the T cell differentiating cocktails caused a portion of the $CD34^+$ cells to differentiate into mature T cells capable of responding to mitogenic stimulation.

FIGURE 5 shows a study similar to that shown in FIGURE 4, however the lymphocytes were stimulated by allo-antigens. Lymphocyte response to stimulation by allo-antigen is MHC-restricted but is not antigen specific at the TCR receptor. Cocktails No. 3, 4, 5, and 6, showed increasing levels of responsiveness to allo-antigen compared to LNMCs from cytokine combinations Nos. 1 (control) or 2.

FIGURE 6 shows a study similar to that shown in FIGURE 4, however, the antigens used in FIGURE 6 are thought to stimulate different populations of T cells. *Staphylococcus aureus* Enterotoxin-A (SE-A) stimulates V chain families 1, 3, 10, 11, and 17, while *Staphylococcus aureus* Enterotoxin-B (SE-B) stimulates V chain families 3, 7, 8,

and 17. Accordingly, the results of this experiment permit one to assess which V chain families were represented in the mature T cells including those differentiated in culture.

Examining the data shown in FIGURE 6, in the first column (Control) is the positive control obtained from the same HIV negative subject as was used in FIGURE 4. The second column (LNMC d-1) reports the Stimulation Index of the LN material on day 1 of the experiment. The third column (LN d80#1) reports the Stimulation Index of a $CD34^+$/LN material coculture on day 80 where the cells were grown in the presence of T cell differentiating cytokine Cocktail No. 1. Cells from this culture responded well to SE-A but showed almost no response to SE-B stimulation (Vβ 7, 8 cells were not present or not responding in the tested culture). A pattern of skewed responsiveness is noted during advanced HIV infection. The Stimulation Index from cells in column 4 (LNd80#2) showed a response to both SE-A and SE-B stimulation, although this response was well below that of the positive control. In contrast, the results shown in columns 5 and 6 (LNd80#3 and LNd80#4, respectively) showed antigen induced stimulation response comparable to those of the positive control. Cocktail No. 3 produced cells that responded with a (50) Stimulation Index reading, as compared to the positive control of slightly more than (50). The SE-B only stimulated a level of (31) as compared to the (47) of the positive control. The response from the cells grown in Cocktail No. 4 gave a Stimulation Index response of approximately (40) for SE-A stimulation and approximately (38) for SE-B. Cocktail No. 5 produce SE-A stimulation of approximately the same magnitude as that of the positive control. Interestingly, the stimulation response for the SE-B was only slightly higher than that of the response seen from the LN material initially isolated, the p24 Ag expression in this sample was increased, perhaps altering the population of T cells present. Cocktail No. 6 produced roughly equal responses for both SE-A and SE-B, although the magnitude of the response was lower than that seen for Cocktail No. 4 or the Control.

The results shown in FIGURE 6 indicate that T cell differentiation cytokine cocktails 2-6 all produced cells that either differentiated into new T cells capable of responding to superantigens, as cocktail No. 1 would only rescue existing T cells and potentially restore their ability to respond to antigenic stimulation.

Example 9

Reconstitution of a Human Immune System Damaged by HIV

A human subject with an immune system compromised by infection with HIV is treated with the methods described above. Autologous lymphoid tissue is isolated according to the methods described above. Similarly, $CD34^+$ cells that are free of the HIV virus are isolated and purified according to the teachings of the present method. The isolated $CD34^+$ cells are cocultured with the autologous lymphoid tissue in the presence of a cytokine cocktail containing IL-2, IL-6, IL-7, OSM, SCF, and flt 3. The cells are cocultured as described above. Samples of the coculture are taken on days 1 (prior to the addition of CD34+ progenitor cells), 14, 45, and 60 to determine the extent to which T cell differentiation has occurred using the assay methods described herein.

Once a population of mature T cells is obtained from the LN material/CD34+ progenitor cell coculture, the mature T cells are administered to the subject so as to supply the subject with viable, HIV-free T cells. The

administration of the cocultured T cells occurs subsequent to an aggressive antiviral chemotherapeutic regime to protect the cocultured HIV-free T cells from infection.

Example 10

Reconstitution of a Human Immune System Damaged by Chemotherapy

A subject with an immune system damaged by chemotherapy is treated with the methods of the present invention to provide the subject with a viable population of mature T cells. Following a course of chemotherapy a population of mature T cells is generated from $CD34^+$ stem cells and lymphoid tissue as described above. Mature T cells are isolated from the coculture and administered to the subject. A course of hemopoietic differentiation stimulating compounds such as EPO are also administered at efficacious concentrations to stimulate the generation of erythroid cells.

Example 11

Reconstitution of a Human Immune System Damaged by Radiotherapy

A subject whose immune system is damaged by radiotherapy undergoes the therapy of the present method. In this case, $CD34^+$ cells are acquired from a donor shown to be compatible with the host patient's immune system. The exogenous stem cells are differentiated according to the methods described above. The result is a patient with a reconstituted immune system.

Example 12

Generating $CD34^+$ Progenitor Cells with an Antisense Tat/Rev HIV Sequence

In this Example mature T cells are generated from $CD34^+$ stem cells according to the methods of the present invention to be introduced into a patient suffering from HIV. $CD34^+$ stem cells are isolated from the patient as described above. Into the isolated cells is introduced an expression vector containing an inverted segment of the HIV genome. The segment corresponds to a fragment of Exon I of the *tat* gene with the sequence 5'-CCAGGAAGUCAGCCUAAAA-3' (SEQ ID NO. 3). Alternatively, an antisense to the *tr* gene sequence 5'-CAGACUCAUCAAGCUUCUC-3' (SEQ ID NO. 4) may be used. In still another alternative, a nucleic acid sequence encoding a ribozyme corresponding to SEQ ID NO. 3 can be used. An example of such a ribozyme is 5'-GGUCCUUCAAAGCAGGAGUGCCUGAGUAGUCUCGGAUUUU-3' (SEQ ID NO. 5). Another embodiment of such a ribozyme is 5'-GUCUGAGUAAAGCAGGAGUGCCUGAGUAGUCUUCGAAGAG-3' (SEQ ID NO. 6). The ribozymes discussed above are disclosed in U.S. Patent No. 5,695,938.

The expression vector is introduced using standard molecular biology techniques well known in the art. The expression vector also contains a drug selection marker, such as the tetracycline resistance marker, so that cells containing a functional copy of the expression vector may be selected for using tetracycline selection.

Twenty-four hours after the expression vector is introduced into the $CD34^+$ cells, the cells are subjected to tetracycline selection. Accordingly, an effective amount of tetracycline is added to the culture media and the cells are grown for an additional 24 hour period. Those cells that survive the selection are then introduced to conditioned

lymph node cells as discussed above. Mature T cells produced from the process are then administered to the HIV infected patient.

Mature CD4$^+$ T cells are vulnerable to HIV infection. The mature CD4$^+$ cells generated in this Example are prevented from producing virus because they contain the expression vector with the *tat* antisense sequence. This sequence prevents the expression of this essential viral protein. Accordingly, the mature CD4$^+$ cells administered to the HIV patient are protected from HIV infection.

Example 13

Local Administration of Cytokines to Secondary Lymphoid Tissue to Expand the Microenvironment Which Facilitates T Cell Lymphopoiesis

In this example a cocktail of T cell differentiating cytokines is administrated to lymphoid tissue in the subject to be treated. The cocktail of T cell differentiating cytokines contains: IL-2, IL-6, IL-7, OSM, SCF, and flt 3. The cocktail is administered by injection according to basic surgical techniques well known in the art. Specifically, a catheter is introduced through the skin and positioned in a lymph node located under the right arm of the subject. Following insertion of the catheter, the cocktail is administered.

The components of the cytokine cocktail function to convert the tissue of the lymph node into a microenvironment in which migrating CD34$^+$ stem cell differentiate into mature T cells.

Example 14

Transplantation of Secondary Lymphoid Tissue

In this example, a patient suffering from complete DiGeorges Syndrome (Congenital Athymia) is transplanted with lymphoid tissue treated with T cell differentiating cytokines. In the first step a lymph node from the subject to be treated is removed using standard surgical techniques. The lymph node, as an intact sample, is cultured for one week in the presence of a T cell differentiating cocktail of cytokines consisting of IL-2, IL-6, IL-7, OSM, SCF, and flt 3. Following this culture period the lymph node is replaced in the body of the subject. The subject then receives regular injections of the T cell differentiating cocktail of cytokines. The conditioned lymph node is replaced in the body of the subject to provide a microenvironment in which CD34$^+$ cells can differentiate into mature T cells.

Conclusion

While the present invention has been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention.

All patents, patent applications and publications referred to above are hereby incorporated by reference.

Sequence Listing

SEQ ID NO. 1: Primer Sequence:
5'-GGCACACCCTTTCCTTCTCTG-3'

SEQ ID NO. 2: Primer Sequence:
5'-GCAGGTCCTGGCTGTAGAAGC-3'

SEQ ID NO. 3: *tat/rev* Exon I of HIV
5'-CCAGGAAGUCAGCCUAAAA-3'

SEQ ID NO. 4: *tr* gene sequence fragment
5'-CAGACUCAUCAAGCUUCUC-3'

SEQ ID NO. 5: HIV ribozyme sequence
5'-GGUCCUUCAAAGCAGGAGUGCCUGAGUAGUCUCGGAUUUU-3'

SEQ ID NO. 6: HIV ribozyme sequence
5'-GUCUGAGUAAAGCAGGAGUGCCUGAGUAGUCUUCGAAGAG-3'

WHAT IS CLAIMED IS:

1. A method for *in vitro* T cell production, comprising:

culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell.

2. The method of claim 1, wherein the hematopoietic T cell progenitor cell is a human cell.

3. The method of claim 2, wherein the hematopoietic T cell progenitor cell is a $CD34^+$ cell.

4. The method of claim 1, wherein the hematopoietic T cell progenitor cell is a simian cell.

5. The method of claim 1, wherein the lymph node cell is a human cell.

6. The method of claim 1, wherein the lymph node cell is a simian cell.

7. The method of claim 1, wherein the hematopoietic T cell progenitor cell is isolated from a subject infected with an immunodeficiency virus.

8. The method of claim 7, wherein the immunodeficiency virus is a human immunodeficiency virus.

9. The method of claim 8, wherein the immunodeficiency virus is human immunodeficiency virus type 1.

10. The method of claim 1, further comprising culturing the hematopoietic T cell progenitor cell in the presence of a cytokine.

11. The method of claim 10, wherein the cytokine is selected from the group consisting of IL-2, IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), stem cell factor (SCF), thrombopoietin (TPO), and flt 3.

12. The method of claim 10, wherein the cytokine comprises at least IL-2 and IL-6.

13. The method of claim 12, wherein the cytokine further comprises leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, IL-7, and thrombopoietin (TPO).

14. The method of claim 12, wherein the cytokine further comprises oncostatin M (OSM), stem cell factor (SCF), flt 3, and IL-7.

15. The method of claim 12, wherein the cytokine further comprises oncostatin M (OSM).

16. The method of claim 12, wherein the cytokine further comprises leukemia inhibitory factor (LIF).

17. The method of claim 12, further comprising isolating the hematopoietic T cell progenitor cell or descendant thereof from the lymph node cell following culturing.

18. The method of claim 12, further comprising:

culturing the progenitor cell and the lymph node cell in the presence of an agent capable of genetically altering the hematopoietic T cell progenitor cell.

19. The method of claim 12, wherein the agent is a virus.

20. The method of claim 12, wherein the virus is a retrovirus.

21. The method of claim 12, further comprising isolating a genetically altered hematopoietic T cell progenitor cell or a descendant thereof.

22. A method for testing the effect of an agent on a hematopoietic cell, comprising: co-culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell;

contacting the hematopoietic T cell progenitor cell with an agent; and

comparing the differentiation or growth of the hematopoietic T cell progenitor cell with the differentiation or growth of a control cell not contacted with the agent.

23. The method of claim 22, wherein the hematopoietic T cell progenitor cell is a human cell.

24. The method of claim 22, wherein the hematopoietic T cell progenitor cell is a $CD34^+$ cell.

25. The method of claim 22, wherein the hematopoietic T cell progenitor cell is a simian cell.

26. The method of claim 22, wherein the lymph node cell is a human cell.

27. The method of claim 22, wherein the lymph node cell is a simian cell.

28. The method of claim 22, wherein the progenitor cell is a genetically altered progenitor cell.

29. The method of claim 22, further comprising culturing the hematopoietic T cell progenitor cell with the lymph node cell in the presence of a cytokine.

30. The method of claim 29, wherein the cytokine comprises at least IL-2 and IL-6.

31. The method of claim 29, wherein the cytokine further comprises leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, IL-7, and thrombopoietin (TPO).

32. The method of claim 29, wherein the cytokine further comprises oncostatin M (OSM), stem cell factor (SCF), flt 3, and IL-7.

33. The method of claim 29, wherein the cytokine further comprises oncostatin M (OSM).

34. The method of claim 29, wherein the cytokine further comprises leukemia inhibitory factor (LIF).

35. A method for modifying a hematopoietic stem cell to produce a descendent cell containing a nucleotide sequence of interest, the method comprising:

culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell to produce a descendent cell;

contacting said primate hematopoietic T cell progenitor cell and said lymph node cells with an expression vector comprising a nucleotide sequence of interest, wherein said contacting is for a period of time sufficient for said virus to enter said hematopoietic T cell progenitor cell.

36. The method of claim 35, wherein said expression vector is a viral vector.

37. The method of claim 36, wherein said viral vector is a retroviral vector.

38. The method of claim 35, wherein the hematopoietic T cell progenitor cell is a human cell.

39. The method of claim 35, wherein the hematopoietic T cell progenitor cell is a $CD34^+$ cell.

40. The method of claim 35, wherein the hematopoietic T cell progenitor cell is a simian cell.

41. The method of claim 35, further comprising culturing the hematopoietic T cell progenitor cell with the lymph node cell in the presence of a cytokine.

42. The method of claim 41, wherein the cytokine comprises at least IL-2 and IL-6.

43. The method of claim 41, wherein the cytokine further comprises leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, IL-7, and thrombopoietin (TPO).

44. The method of claim 41, wherein the cytokine further comprises oncostatin M (OSM), stem cell factor (SCF), flt 3, and IL-7.

45. The method of claim 41, wherein the cytokine further comprises oncostatin M (OSM).

46. The method of claim 41, wherein the cytokine further comprises leukemia inhibitory factor (LIF).

47. A method of treating a subject having an immune disorder, comprising

culturing a lymph node cell *in vitro* with a primate hematopoietic T cell progenitor cell to produce a descendent cell;

administering a therapeutically effective amount of said progenitor cell or said descendent of said progenitor cell to said subject.

48. The method of claim 47, wherein the immune disorder is an immune deficiency disorder.

49. The method of claim 47, wherein the immune disorder is an immunoproliferative disorder.

50. The method of claim 47, wherein the hematopoietic T cell progenitor cell is a human cell.

51. The method of claim 50, wherein the hematopoietic T progenitor cell is a $CD34^+$ cell.

52. The method of claim 47, wherein the hematopoietic T cell progenitor cell is a simian cell.

53. The method of claim 47, wherein the lymph node cell is a human cell.

54. The method of claim 47, wherein the lymph node cell is a simian cell.

55. The method of claim 47, wherein said hematopoietic T cell progenitor cell is isolated from a subject infected with an immunodeficiency virus.

56. The method of claim 55, wherein said immunodeficiency virus is a human immunodeficiency virus.

57. The method of claim 56, wherein said human immunodeficiency virus is human immunodeficiency virus type 1.

58. The method of claim 47, further comprising culturing the hematopoietic T cell progenitor cell in the presence of a cytokine.

59. The method of claim 53, wherein the cytokine is selected from the group consisting of IL-2, IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), stem cell factor (SCF), thrombopoietin (TPO), and flt 3.

60. The method of claim 53, wherein the cytokine comprises at least IL-2 and IL-6.

61. The method of claim 53, wherein the cytokine further comprises leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, IL-7, and thrombopoietin (TPO).

62. The method of claim 53, wherein the cytokine further comprises oncostatin M (OSM), stem cell factor (SCF), and IL-7.

63. The method of claim 53, wherein the cytokine further comprises oncostatin M (OSM).

64. The method of claim 53, wherein the cytokine further comprises leukemia inhibitory factor (LIF).

65. A method of treating an immune disorder in a subject, comprising administering a therapeutically effective amount of a cytokine to a lymph node in said subject, wherein said cytokine comprises at least IL-2 and IL-6.

66. The method of claim 65, wherein the cytokine further comprises leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, IL-7, and thrombopoietin (TPO).

67. The method of claim 65, wherein the cytokine further comprises oncostatin M (OSM), stem cell factor (SCF), flt 3, and IL-7.

68. The method of claim 65, wherein the cytokine further comprises oncostatin M (OSM).

69. The method of claim 65, wherein the cytokine further comprises leukemia inhibitory factor (LIF).

70. The method of claim 65, wherein said administering is systemic.

71. A kit comprising:

a container containing a cryopreserved lymph node cell; and

instructions for culture of said lymph node cell with a hematopoietic T cell progenitor cell.

72. The kit of claim 72, further comprising at least one container containing a cytokine.

73. The kit of claim 73, wherein said cytokine comprises at least IL-2 and IL-6.

74. The kit of claim 74, wherein the cytokine further comprises leukemia inhibitory factor (LIF), stem cell factor (SCF), flt 3, IL-7, and thrombopoietin (TPO).

75. The kit of claim 74, wherein the cytokine further comprises oncostatin M (OSM), stem cell factor (SCF), flt 3, and IL-7.

76. The kit of claim 74, wherein the cytokine further comprises oncostatin M (OSM).

77. The kit of claim 74, wherein the cytokine further comprises leukemia inhibitory factor (LIF).

78. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least one member selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

79. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least two members selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

80. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least three members selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

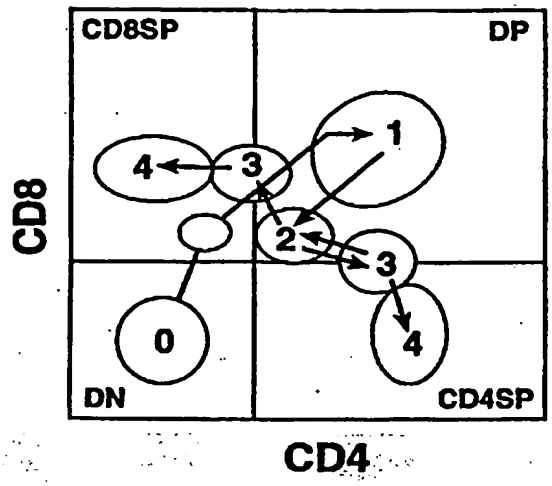
81. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least four members selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

82. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least five members selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

83. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least six members selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

84. The method of Claim 10, 29, 41, 58 or the kit of Claim 73, wherein said cytokine is at least seven members selected from the group consisting of IL-2, IL-6, IL-7, OSM, LIF, SCF, flt 3, and TPO.

FIGURE 1



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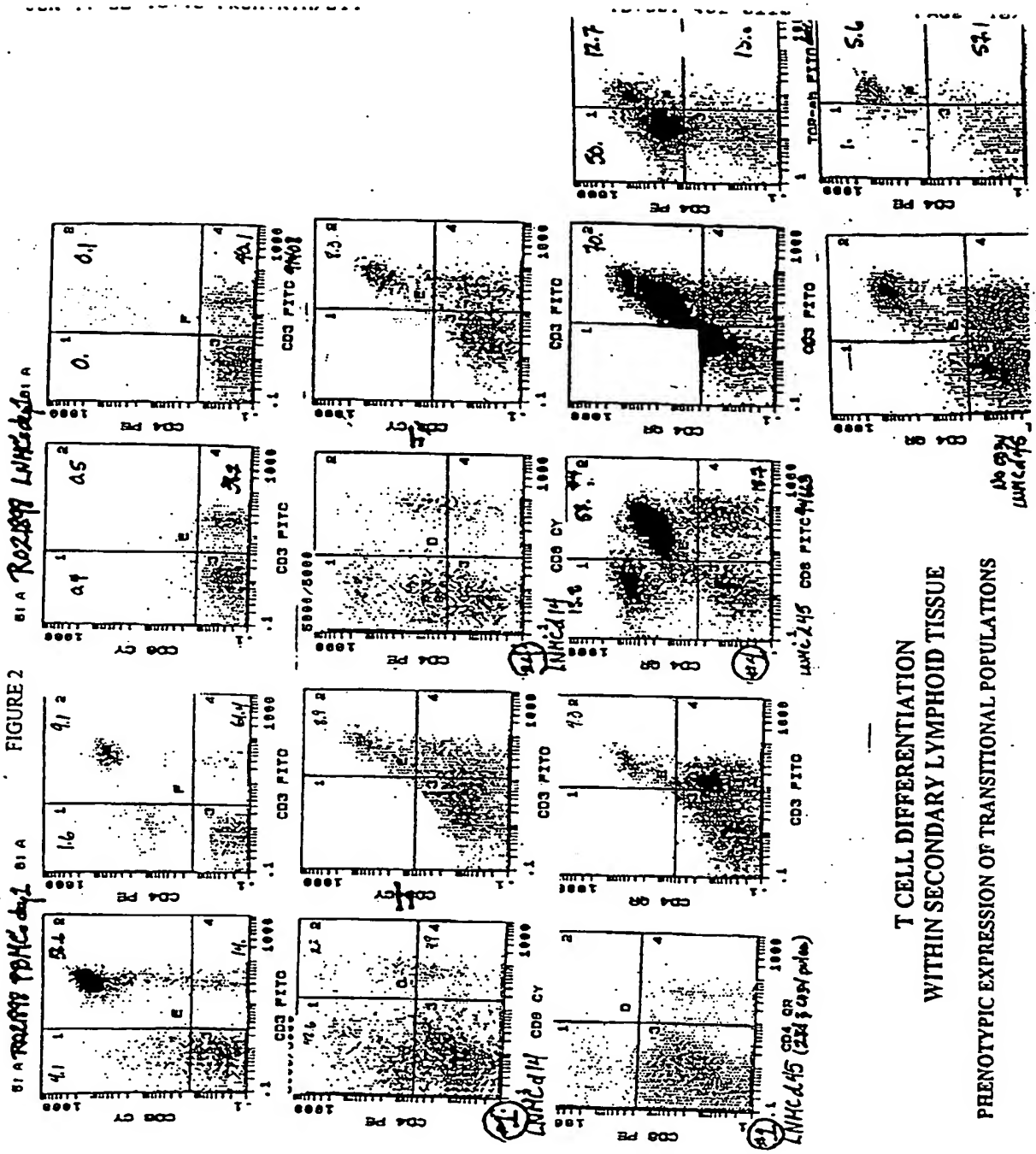

FIGURE 2

T CELL DIFFERENTIATION
WITHIN SECONDARY LYMPHOID TISSUE

PHENOTYPIC EXPRESSION OF TRANSITIONAL POPULATIONS

HIV+LNF Cocultured With Autologous CD34+ Progenitor Cells

PCT/US 00/29655

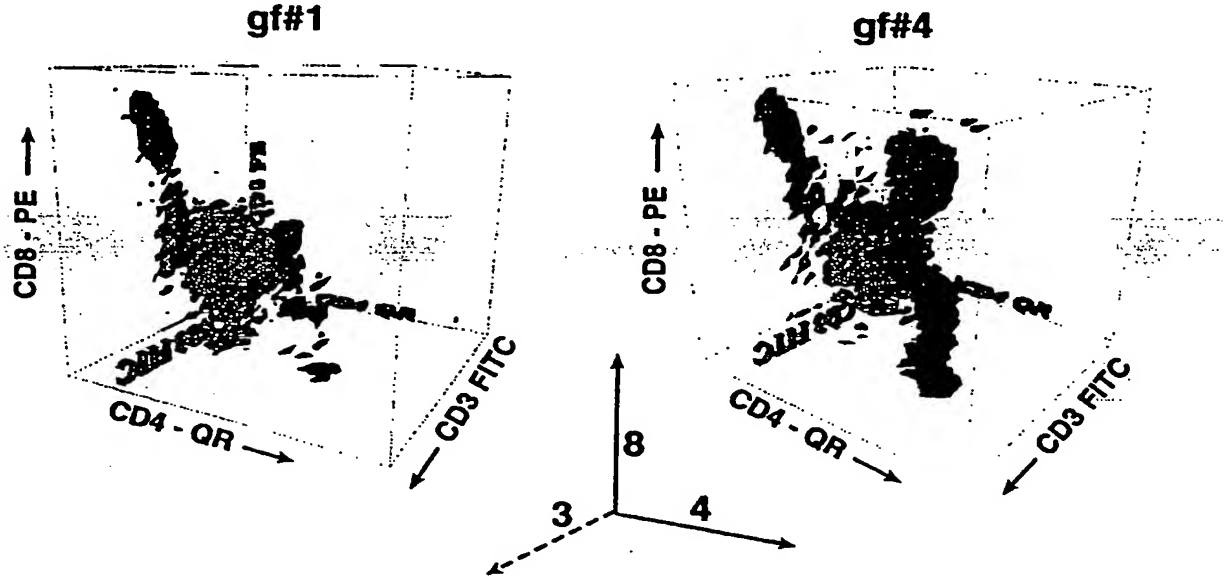

FIGURE 3

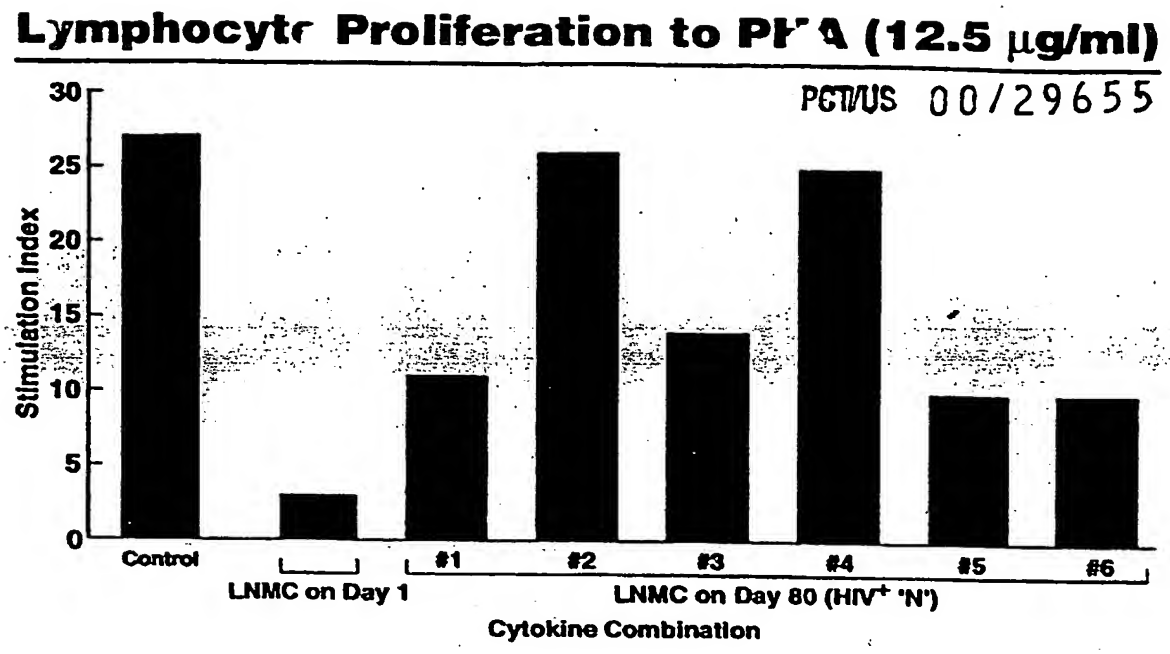
Lymphocyte Proliferation to PHA (12.5 µg/ml)
PCT/US 00/29655
Stimulation Index
30
25
20
15
10
5
0
Control
#1
#2
#3
#4
#5
#6
LNMC on Day 1
LNMC on Day 80 (HIV+ 'N')
Cytokine Combination

FIGURE 4

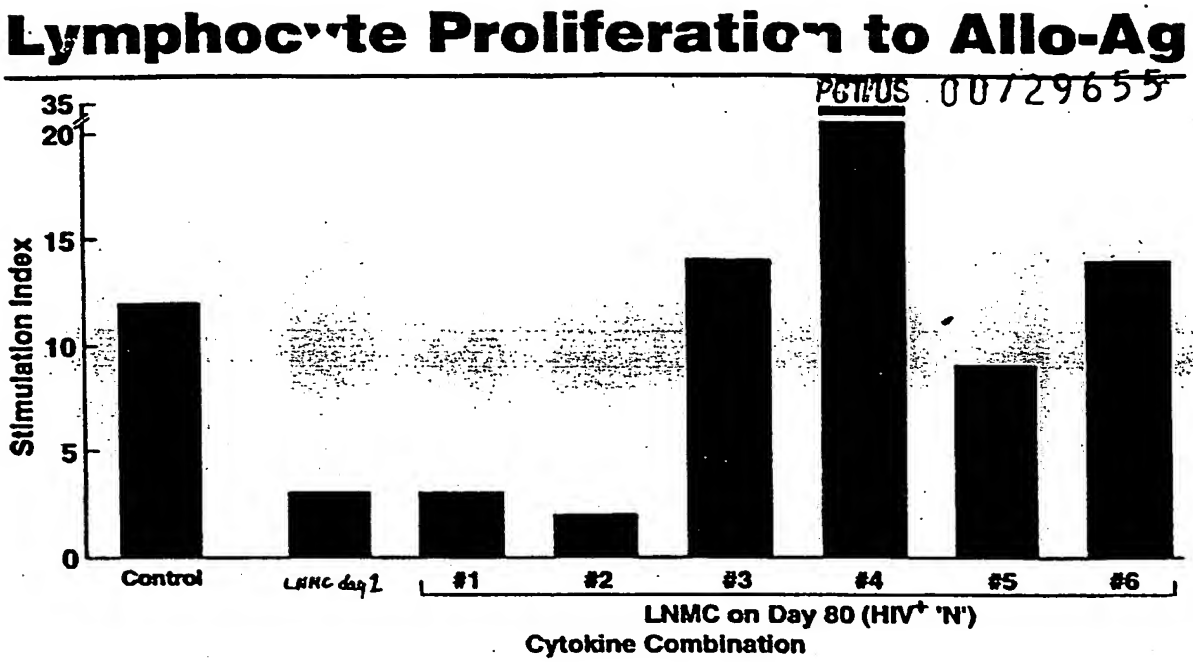
Lymphocyte Proliferation to Allo-Ag
PCT/US 00/29655
Stimulation Index
35
20
15
10
5
0
Control
LNMC day 1
#1
#2
#3
#4
#5
#6
LNMC on Day 80 (HIV+ 'N')
Cytokine Combination

FIGURE 5

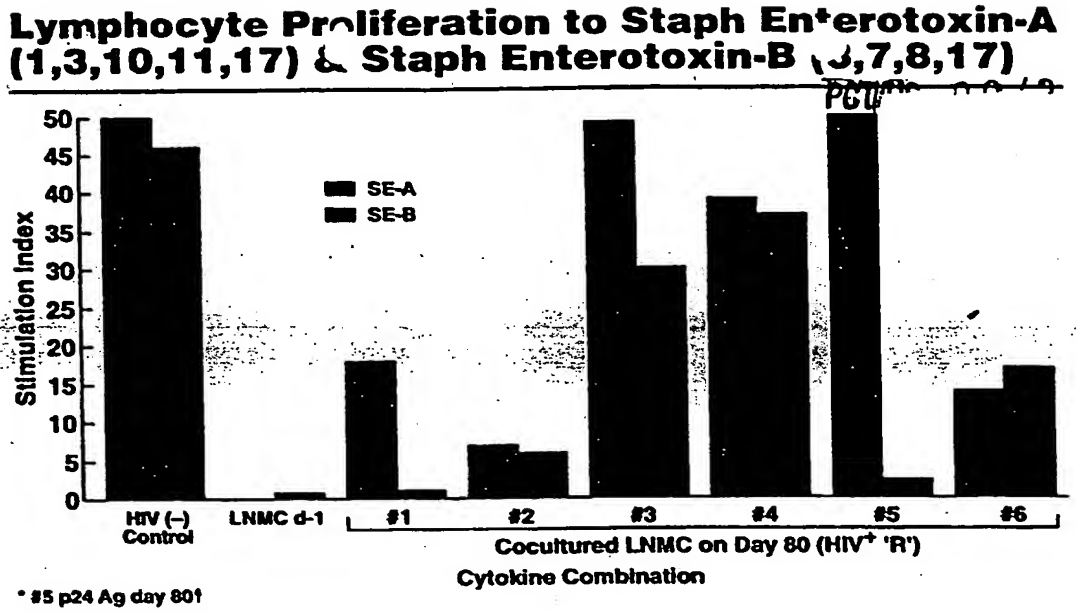
Lymphocyte Proliferation to Staph Enterotoxin-A (1,3,10,11,17) & Staph Enterotoxin-B (3,7,8,17)
Stimulation Index
50
45
40
35
30
25
20
15
10
5
0
SE-A
SE-B
HIV (–) Control
LNMC d-1
#1
#2
#3
#4
#5
#6
Cocultured LNMC on Day 80 (HIV+ 'R')
Cytokine Combination
* #5 p24 Ag day 80†

FIGURE 6